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Extremely Thermostable D-Glyceraldehyde-3-phosphate Dehydrogenase from the Eubacterium *Thermotoga maritima*†

Alexander Wrba, Anita Schweiger, Verena Schultes, and Rainer Jaenicke*
Institut für Biophysik und Physikalische Biochemie der Universität Regensburg, D-8400 Regensburg, FRG
Péter Závodszky
Institute of Enzymology, Biological Research Centre, Hungarian Academy of Sciences, Budapest, Hungary
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ABSTRACT: D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Thermotoga maritima*, a hyperthermophilic eubacterium, has been isolated in pure crystalline form. The enzyme is a homotetramer with a subunit molecular mass of 37 kDa. The sedimentation coefficient of the native enzyme is 7.3×10^{-13} s, the isoelectric point is 4.6, and the specific absorption coefficient $A_{280nm}^{1\%,\,1\,cm} = 8.4$. The enzyme shows extreme thermal stability: differential scanning calorimetry yields a transition temperature (T_m) of 109 °C for the NAD-saturated enzyme. Thermal deactivation occurs at $T > 90$ °C. The physicochemical characteristics of the enzyme suggest that its gross structure must be very similar to the structure of GAPDHs from mesophilic sources. The amino acid composition does not confirm the known "traffic rules" of thermal adaptation, apart from the Lys → Arg exchange. One reactive and at least two buried SH groups can be titrated with 5,5′-dithiobis(2-nitrobenzoate). The highly reactive SH group is probably the active-site cysteine residue common to all known GAPDHs. The activation energy of the glyceraldehyde 3-phosphate oxidation reaction decreases with increasing temperature. This functional behavior can be correlated with the temperature-dependent changes of both the intrinsic fluorescence and the near-UV circular dichroism; both indicate a temperature-dependent structural reorganization of the enzyme. Hydrogen–deuterium exchange reveals significantly increased rigidity of the thermophilic enzyme if compared to mesophilic GAPDHs at 25 °C, thus indicating that the conformational flexibility is similar at the corresponding physiological temperatures. The increase in $\overline{\Delta G}$, i.e., the Gibbs energy of the average microunfolding exposing peptide hydrogens to the solvent, is 5.2 kJ/mol, going from the mesophilic to the thermophilic enzyme. The effect may be attributed to the increased saturation of the structure with nonpolar contacts.

Glyceraldehyde-3-phosphate dehydrogenases (GAPDHs,[1] EC 1.2.1.12) have been isolated from a variety of species (Harris & Waters, 1976), including mesophilic (Krebs et al., 1953; d'Alessio & Josse, 1971; Misset et al., 1987), moderately thermophilic (Amelunxen, 1966; Hocking & Harris, 1973; Fujita et al., 1976; Crabb et al., 1977), and (hyper)thermophilic microorganisms (Hensel et al., 1987; Fabry & Hensel, 1987). The latter enzymes show increased thermal stability and are enzymatically active at elevated temperatures.

We observed a high level of GAPDH activity in the cell homogenate of *Thermotoga maritima*. This microorganism is an extremely thermophilic eubacterium, isolated from geothermally heated locales on the sea floor (Huber et al., 1986). It grows optimally between 80 and 90 °C. Our preliminary studies indicated that the enzyme is remarkably stable, even at 100 °C, thus representing the most stable glycolytic enzyme isolated so far. This extreme thermostability opens a way to study enzyme action and regulation, as well as protein folding in an extended temperature range.

A series of homologous GAPDHs from various sources, with widely differing thermal stabilities, have been shown to be

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* Address correspondence to this author at Biochemie II, Universität Regensburg, Universitätsstrasse 31, D-8400 Regensburg, FRG.

[1] Abbreviations: GAPDH, D-glyceraldehyde-3-phosphate dehydrogenase; EDTA, ethylenediaminetetraacetic acid; TEA, triethanolamine; CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTE, dithioerythritol; PEG, poly(ethylene glycol); DSC, differential scanning calorimetry.

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highly similar in their sequence, subunit composition, and enzymatic behavior (Harris & Waters, 1976). By comparing these homologues, it might be possible to clarify the mechanism of adaptation to extreme environmental conditions at the molecular level.

Protein stability is commonly expressed in terms of the energy required for the micro- and macrounfolding of the structure of a given protein, as determined from hydrogen exchange and denaturation experiments. Both these characteristics represent qualitatively different properties of a protein molecule which can be classified (i) as the rigidity of the structure, and (ii) as the stability of the macroscopic state (Privalov & Tsalkova, 1979).

The extremely thermophilic variant of a well-characterized enzyme provides an opportunity to study the mechanism of how nature adjusts protein structure to extreme environmental conditions, keeping the delicate balance between stability and conformational flexibility.

Materials and Methods

Chemicals. DEAE-cellulose (DE 52) and phenyl-Sepharose were purchased from Whatman (Maidstone) and Pharmacia (Uppsala), respectively. For affinity chromatography, CH-Sepharose 4B–N^6-(2-aminoethyl)-NAD was used; the material was kindly provided by A. F. Bückmann (GBF, Braunschweig).

NAD^+, NADH, ATP, glyceraldehyde 3-phosphate (barium salt), 3-phosphoglyceric acid, yeast phosphoglycerate kinase, and yeast GAPDH were purchased from Boehringer (Mannheim); cysteine hydrochloride was from Sigma (Deisenhofen). All other chemicals were analytical-grade substances from Merck (Darmstadt). Quartz-bidistilled water was used throughout.

Cultivation of the Microorganism. A culture of *Thermotoga maritima* (MSB 8; DSM strain 3109) was kindly provided by K. O. Stetter and R. Huber (Lehrstuhl für Mikrobiologie, Universität Regensburg). Cells were grown as described by Huber et al. (1986) using a 300-L fermentor at 80 °C under N_2 atmosphere. Cells were stored at −80 °C. The yield of cells was about 120 g per 300-L culture.

Isolation of the Enzyme. To purify the enzyme, ammonium sulfate precipitation was followed by hydrophobic, ion-exchange, and affinity chromatography using NAD–Sepharose. For the detailed protocol, see Results.

Enzyme Assay. (*A*) *Oxidation of Glyceraldehyde 3-Phosphate.* Enzyme activity was monitored by tracing the absorbance at 366 nm in an Eppendorf spectrophotometer using 1-cm glass cuvettes sealed with silicone stoppers. Measurements were performed at 20–75 °C. The cuvettes were thermostated, and the reaction mixture was preincubated for 5 min; the actual temperature in the cuvettes was controlled by a thermistor unit.

The standard assay mixture contained 3 mM NAD^+, 10 mM potassium dihydrogen arsenate, and 5.2 mM glyceraldehyde 3-phosphate in 50 mM Teorell–Stenhagen buffer, pH 8.5 (Teorell & Stenhagen, 1939). The same buffer was used to follow the pH dependence of enzymatic activity. The specific activity of the enzyme was routinely measured at 40 °C (standard assay). One activity unit was defined as the amount of GAPDH that catalyzes the formation of 1 μmol of NADH/min at 40 °C.

In order to determine the thermal stability at elevated temperature, the time course of deactivation was measured at varying pH. To exclude reactivation in the standard assay, trypsin was added in control experiments (Jaenicke & Rudolph, 1986). Since glyceraldehyde 3-phosphate is thermally unstable, it was added right before the reaction was started by the enzyme. The activity was calculated from the initial velocity of the reaction. At higher temperatures, blank experiments in the absence of the enzyme were performed in order to correct for NADH autoxidation. The oxidized coenzyme, NAD^+, is stable up to 70 °C so that no correction was required (Wrba, 1989).

(*B*) *Reduction of 1,3-Bisphosphoglycerate.* The enzymatic reduction of 1,3-bisphosphoglycerate was also followed spectrophotometrically at 366 nm. The assay mixture contained 0.26 mM NADH, 5 mM cysteine hydrochloride, 3.2 mM 3-phosphoglyceric acid, 1.1 mM ATP, 6.5 mM EDTA, and 10 μg of yeast 3-phosphoglycerate kinase in 50 mM TEA-HCl buffer, pH 7.6, plus 5 mM EDTA (total volume 1 mL). Because of the instability of the yeast enzyme and of 1,3-bisphosphoglycerate, this assay could only be used at temperatures below 50 °C. Standard measurements were carried out at 40 °C; pH measurements refer to this reference temperature, K_m values were obtained from fitted linear Lineweaver–Burk plots.

Amino Acid Analysis. Protein samples were hydrolyzed in 5.7 M twice-distilled HCl at 110 °C for 24 h. For determination of the isoleucine and valine content of the undigested protein, the hydrolysis was prolonged to 120 h. Amino acids were analyzed in Kontron Ligemat III and Beckman 6 300 amino acid analyzers using ion-exchange separation and ninhydrin postcolumn detection according to Spackman et al. (1958). SH groups were titrated with 5,5′-dithiobis(2-nitrobenzoate) using the enzyme in its native state and in 8 M urea. The titrations were made in 50 mM Tris-HCl buffer, pH 8.0, plus 1 mM EDTA. The procedure of Janatova et al. (1968) was followed.

Protein Determination. The extinction coefficient of GAPDH from *Thermotoga maritima* at 280 nm was determined by using a Brice Phoenix differential refractometer at 546 nm. The refractive index increment at 546 nm, $\Delta n/c$ = 0.184 mL/g, was used with yeast GAPDH as a standard.

SDS–Polyacrylamide Slab Gel Electrophoresis. Protein samples were prepared according to Laemmli (1970); 8–25% gradient gels were used in a Phast-System (Pharmacia, Uppsala). Gels were stained with silver or Coomassie Brilliant Blue following procedures recommended by the manufacturer.

Ultraviolet Absorption Spectroscopy. Cary 118 and Perkin-Elmer 551S double-beam spectrophotometers were used with thermostated quartz cuvettes. The temperature in the cuvette was checked by using a thermistor telethermometer. The samples were centrifuged in an Eppendorf centrifuge at 10000*g* before the measurements.

Fluorescence Emission Spectra. Fluorescence emission spectra were recorded with a Perkin-Elmer MPF 44A spectrofluorometer equipped with a corrected spectra computer. In order to increase the precision of localization of the fluorescence maxima, λ_{max}, the midpoint between the 80% intensities on both edges of the fluorescence peak was used. The excitation wavelength was 280 nm, the excitation bandwidth 2 nm, and the emission bandwidth 5–8 nm.

Circular Dichroism. A Jasco J 500 A circular dichroism spectropolarimeter with a 500 N data processor was used, with 0.1-, 0.2-, and 1.0-cm thermostated quartz cuvettes. The samples were centrifuged at 10000*g* before the measurements. The mean residue ellipticities were determined by using a mean residue weight of 110.

Hydrogen–Deuterium Exchange. The time course of hydrogen–deuterium exchange of the peptide hydrogens was followed by infrared spectroscopy, as described earlier (Hvidt

& Nielsen, 1966; Závodszky et al., 1975). In order to transfer the enzyme from ^1H_2O into the appropriate ^2H_2O buffer, protein solutions (40 mg/mL) were applied to a G-25 superfine Sephadex column (0.4 × 8 cm). Half of the 0.15-mL samples was saved to measure pH* and enzymatic activity; the other half was filled into a 100-μm CaF_2 cell. ^2H_2O buffer was filled into the reference cell. The temperature was maintained at 25.0 ± 0.1 °C. The pH* values specified for the exchange experiments in ^2H_2O were direct readings on the pH meter. The absorption at the maximum of the amide II band at 1544 cm^{-1} (associated with the –CONH– groups) was recorded as a function of time. Scans were started about 2 min after having transferred the protein into ^2H_2O and continued for 6–8 h. At 2-h intervals, the spectral region of 1300–1800 cm^{-1} was scanned. The absorbance at the maximum of the amide I bond at 1640 cm^{-1} (due to the peptide C=O group) was used as internal reference of protein concentration. X, the fraction of unexchanged peptide hydrogen atoms, was determined as

$$X = \frac{A_{\text{amideII}} - A_{\text{amideII},\infty}}{\omega A_{\text{amideI}}} \quad (1)$$

i.e., the ratio of absorbances at the maximum of amide II and amide I bands. ω is this ratio for the undeuterated protein and was determined by extrapolation to zero time of kinetic measurements of the $A_{\text{amideII}}/A_{\text{amideI}}$ ratio at low temperature and low pH. $A_{\text{amideII},\infty}$ is the absorbance of the completely deuterated protein sample at 1544 cm^{-1}. The extrapolated value of ω for GAPDH from *Thermotoga maritima* is found to be 0.46 ± 0.04. In order to determine the actual exchange, the amide II absorption after complete deuteration of the peptide groups was subtracted from the measured amplitudes. To estimate this absorption, ^2H_2O solutions containing 3% SDS at the corresponding pH were heated to 80 °C for 24 h; subsequently, spectra were scanned at 25 °C. Since the presence of SDS slightly reduces the contribution of COO^- to the absorption at 1544 cm^{-1}, the background is underestimated by a few percent. SDS was required because complete deuteration of the hyperthermophilic enzyme cannot be achieved in the absence of detergents or other denaturing agents.

Ultracentrifugal Analysis. Sedimentation velocity and sedimentation equilibrium measurements were performed in a Beckman Spinco Model E analytical ultracentrifuge equipped with a high-intensity light source according to Flossdorf (GBF, Braunschweig) and a photoelectric scanning system. A MOM type 3170/b analytical ultracentrifuge equipped with schlieren and Rayleigh interferometer optics was also used. Sedimentation velocity experiments were run between 44 000 and 60 000 rpm. Sedimentation coefficients were corrected to 20 °C and water viscosity. The equilibrium runs made use of the meniscus depletion technique (Yphantis, 1964) at scanning wavelengths of 230 and 280 nm and rotor speeds of 10 000 and 16 000 rpm. Weight-average molecular weights were determined in the protein concentration range of 3 μg/mL–1.5 mg/mL.

Calorimetric Analysis. Calorimetric measurements were done in a DASM-4 adiabatic differential scanning microcalorimeter (DASM 4, Poushchino). Runs were performed between 15 and 120 °C at a scan rate of 1 °C/min. The protein solutions were dialyzed, and the dialysis buffer was used as a reference. The base line for each run was determined in an identical experiment with buffer in both cells.

Titration of the SH Groups. Modification of SH groups was carried out by reacting the enzyme with 5,5′-dithiobis-(2-nitrobenzoate) at room temperature according to Ellman (1959). The enzyme concentration was 2–6 μM, and a 4–100-fold excess of DTNB was applied in 50 mM Tris-HCl buffer, pH 8.0, plus 1 mM EDTA. Before the titration, the enzyme solutions were freed from DTE by gel filtration on a Sephadex G-25 column. The course of the reaction was followed at 412 nm with a Zeiss M 40 double-beam recording spectrophotometer (C. Zeiss, Jena). Molar extinction coefficients of 14 150 and 14 290 M^{-1} cm^{-1} were applied to determine the amount of thionitrobenzoate ions liberated in pure buffer and buffer in the presence of 8 M urea, respectively (Ellman, 1959; Riddles et al., 1979). In order to obtain the number of rapidly reacting SH groups, the absorbance was extrapolated to zero time from the 1–3-min range of the reaction.

The total number of sulfhydryl groups was determined after incubating the samples in 8 M urea at 60 °C for 2 h. The absorbance of a blank containing buffer and DTNB (plus urea when required) was always subtracted from that of the reaction mixtures.

Isoelectric Focusing. The isoelectric point was determined by isoelectric focusing, using an isoelectric focusing gel with an Ampholine gradient in the pH range of 4.0–6.5, and soybean trypsin inhibitor (p*I* 4.55), β-lactoglobulin A (p*I* 5.20), and carbonic anhydrase B (p*I* 5.85) as standards.

Table I: Purification of GAPDH from *Thermotoga maritima*

step	volume (mL)	act. total units	act. sp units/mg	recovery (%)
crude extract	300	8200	1.5	100
supernatant, 70% ammonium sulfate	280	7000	10.2	85.4
phenyl-sepharose	130	6700	11.0	81.7
DEAE-cellulose	110	5000	41.0	61.0
NAD–Sepharose	11	3520	44.0	42.9

Results

Isolation and Purification of the Enzyme. (A) Crude Extract. Frozen cells (120-g wet weight) were thawed, suspended in 1 L of 50 mM sodium acetate buffer, pH 5.8, plus 1 mM EDTA (buffer A), and washed twice. Washed cells were resuspended in 300 mL of buffer A and disrupted in a French press [(1.0–1.4) × 10^8 N/m^2, i.e., 15–20 000 psi]; 5 mM DTE was added to the crude extract under continuous stirring. Unbroken cells and cell debris were removed by centrifugation (40000*g*, 180 min). The total GAPDH activity in the crude extract was 8200 units, as measured at 40 °C by the 1,3-bisphosphoglycerate reduction reaction. This can be an overestimate because of the presence of other NADH reductase activities (Table I).

(B) Ammonium Sulfate Precipitation. Solid ammonium sulfate was added, under continuous stirring, to bring the supernatant to 70% saturation at 20 °C. After being stirred for 60 min, the precipitated proteins were separated by centrifugation (40000*g*, 90 min).

(C) Hydrophobic Chromatography. The supernatant, 280 mL containing 7000 units of enzymatic activity, was applied to a 100-mL phenyl-Sepharose column (2.5 × 20 cm) and equilibrated with 50 mM Tris-HCl buffer, pH 8.0, plus 1 mM EDTA, 1 mM DTE, and 30% saturated ammonium sulfate. GAPDH activity was quantitatively retained on the column. Elution was accomplished with 50 mM Tris-HCl buffer, pH 8.0, plus 1 mM EDTA and 1 mM DTE (buffer B). A total of 6700 units of enzyme activity were recovered in 130-mL volume. This step was used to remove ammonium sulfate and, at the same time, to decrease the volume.

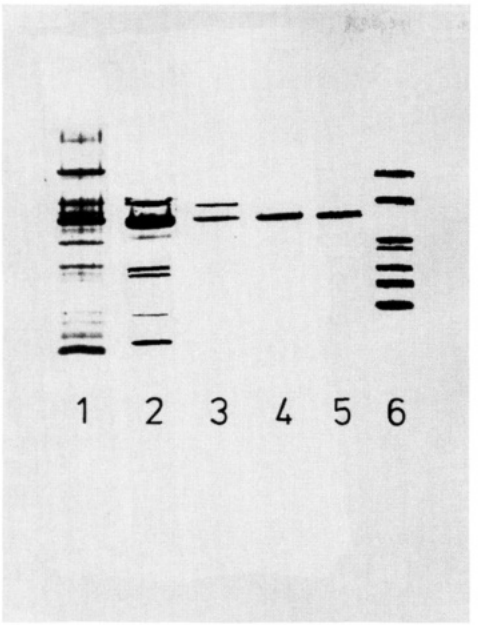

FIGURE 1: SDS–polyacrylamide gel electrophoresis of samples from purification steps of D-glyceraldehyde-3-phosphate dehydrogenase from *Thermotoga maritima*. An 8–25% gradient gel was used. From left to right, (1) crude extract, (2) supernatant of 70% ammonium sulfate, (3) DEAE-52 chromatography, (4) affinity purified, (5) GAPDH from yeast (Boehringer), and (6) Pharmacia molecular mass standard. The gel was silver stained.

(*D*) *Ion-Exchange Chromatography*. The eluate from the phenyl-Sepharose column was dialyzed against buffer B. For further purification, anion exchange on DEAE-cellulose (DE 52) was employed. A 200-mL (4 × 16 cm) column was equilibrated with buffer B, and the dialyzate was applied at a flow rate of 70 mL/h. Quantitative binding was observed. Elution was performed using a pulse of 80 mM NaCl in buffer B; 110 mL of the eluate was collected with a total enzyme activity of 5000 units. The eluate was dialyzed against buffer B.

(*E*) *Affinity Chromatography*. A 9-mL (1.2 × 6 cm) column was prepared with CH-Sepharose 4B–N^6-(2-aminoethyl)-NAD, containing 2.4 μmol of NAD/mL. The column was equilibrated with buffer B. Subsequently, the eluate was divided into two halves which were run separately at a flow rate of 16 mL/h. Out of 2500 units of enzyme, 2250 units were bound to the matrix after the column was washed with 200 mL of buffer B. Elution made use of 10 mM NAD^+ in buffer B, leading to a recovery of 1760 units of GAPDH in 5.5 mL. In the peak fractions, the concentration of the enzyme exceeded 10 mg/mL. For immediate use, the enzyme was stored at 4 °C; for long-term storage, the concentrated enzyme solution was kept in small portions at −70 °C after shock-freezing in liquid nitrogen.

Protein samples after each purification step were analyzed by SDS–polyacrylamide gel electrophoresis under reducing and nonreducing conditions. The purity of the enzyme after the ion-exchange chromatography step was ≥95%. After affinity chromatography, no contamination could be detected even with silver staining. The SDS–polyacrylamide gel electrophoresis patterns are presented in Figure 1.

Crystallization of the Enzyme. Solid ammonium sulfate was added slowly to a 10 mg/mL enzyme solution in buffer B at room temperature until 1.2 M concentration was reached. After the solution was cooled down to 0 °C, saturated ammonium sulfate was added dropwise to bring the solution to slight turbidity. The sample was then allowed to stand for a few hours at room temperature, followed by incubation at 4 °C. Fine crystal needles were formed the next day.

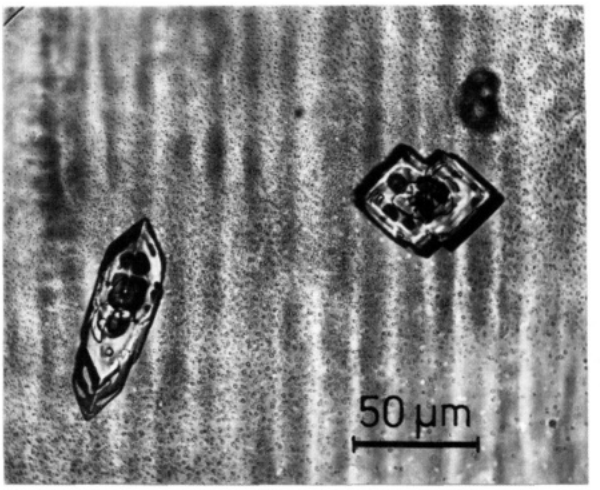

FIGURE 2: Single crystals of GAPDH from *Thermotoga maritima*. Crystallization by the hanging drop method was accomplished in 50 mM Tris-HCl buffer, pH 8.0, in the presence of excess NAD^+ and 1.25% PEG 5000.

The hanging drop method was used to obtain large single crystals; 5.5 mg/mL GAPDH in 50 mM Tris-HCl buffer, pH 8.0, plus 1.25% PEG 5000 and excess NAD^+, was applied as a hanging drop over 1 mL of 50 mM Tris-HCl buffer, pH 8.0, plus 2.5% PEG in the well. After equilibration at 4 °C, single crystals of about 100 × 20 × 20 μm were formed after 4 days (Figure 2).

Molecular Properties of GAPDH from Thermotoga maritima. The molecular mass and subunit composition of the purified enzyme were determined by SDS–polyacrylamide gel electrophoresis and sedimentation analysis. SDS–polyacrylamide gel electrophoresis yields 37 ± 2 kDa. In this experiment, yeast GAPDH with a subunit molecular mass of 36 kDa was used as a reference (cf. Figure 1) (Jaenicke et al., 1968). Sedimentation velocity experiments show that the enzyme sediments as a single symmetrical boundary with a sedimentation coefficient $s_{20,w} = (7.33 \pm 0.05) \times 10^{-13}$ s. Sedimentation equilibrium experiments evaluated at protein concentrations above 80 μg/mL provide clear evidence for a homogeneous tetrameric quaternary structure with a molecular mass of 148 kDa. In the concentration range of 3–90 μg/mL and at high rotor speed, a dissociation–association equilibrium is observed. The extrapolated minimum molecular mass, 37 kDa, calculated at minimum protein concentration is in agreement with the subunit molecular mass estimated from SDS–polyacrylamide gel electrophoresis. The partial specific volume, calculated from the amino acid composition (Table II), is 0.743 $cm^3 \cdot g^{-1}$. Considering the tetramer molecular mass, the measured sedimentation coefficient would be predicted assuming a roughly spherical shape of the protein.

A specific absorption coefficient of $A_{280nm}^{1\%\ 1\ cm} = 8.4$ was obtained from differential refractometry and protein assays according to Bradford (1976) using the yeast enzyme as a reference. The isoelectric point (determined by isoelectric focusing) is 4.6 ± 0.1.

Amino Acid Composition. A comparison of the amino acid composition of GAPDH from *Thermotoga maritima* with amino acid compositions of the enzyme obtained from other species with widely differing stabilities is summarized in Table II. Although it is well-known from previous studies that no simple and unambiguous correlation exists between amino acid composition and thermal stability, certain regularities have been proposed (Argos et al., 1979; Jaenicke, 1981, 1988). Only

Table II: Amino Acid Compositions of D-Glyceraldehyde-3-phosphate Dehydrogenase from *Thermotoga maritima* and Other Sources[a]

amino acid	*Thermotoga maritima*	yeast	thermophiles	mesophiles
Gly	24	27	25–26	26–35
Ala	25	32	38–44	32–34
Val	36	37	28–42	33–38
Leu	26	20	26–31	18–20
Ile	30	19	19–22	18–21
Met	4	6	5–7	6–10
Phe	8	10	5–6	10–15
Trp*	2	3	2–3	2–3
Pro	14	12	11–12	11–13
Ser	15	29	13–18	18–29
Thr	27	23	18–22	18–23
Asx	35	36	34–40	29–39
Glx	26	19	24–28	18–24
Cys**	3	2	1–3	2–5
Tyr	9	11	8–10	9–11
Lys	27	26	23–24	24–28
Arg	14	11	14–16	9–11
His	7	8	9–10	5–11
sum	332	331	333–335	332–336

[a] Asterisks refer to values based on sequence homology (*) and titration with DTNB (**), respectively.

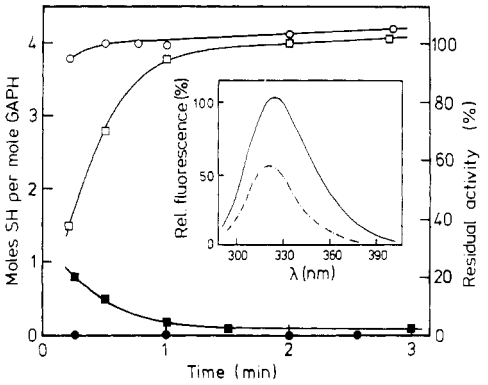

FIGURE 3: Titration of SH groups and inactivation of GAPDH from *Thermotoga maritima*. Enzyme concentration, 4 μM in 50 mM Tris-HCl buffer, pH 8.0; DTNB concentration, 16 μM (□, ■) and 160 μM (○, ●), respectively. Open symbols refer to the number of SH groups modified per mole of tetramer, closed symbols to the residual enzyme activity measured at 40 °C. Insert: fluorescence spectra of the native enzyme (—) and the enzyme after modification of 1 SH/subunit by DTNB (-·-). Excitation wavelength, 280 nm; enzyme concentration, 20 μg/mL in 50 mM sodium phosphate buffer, pH 7.5, 20 °C.

part of these hypothetical strategies of thermal adaptation seem to hold for the present example. In the amino acid analysis, Pro, Cys, and Trp were not determined. However, preliminary sequence data (Schultes et al., 1990), as well as sequence comparison, allow the number of prolines and tryptophans to be estimated. Since both residues have been conserved to a high extent during evolution, 14 prolines and 2 tryptophans per subunit may be assumed for GAPDH from *Thermotoga maritima*.

Cysteine determination made use of the specific titration of thiol groups using 5,5′-dithiobis(2-nitrobenzoate). Experiments were performed using the native enzyme, as well as the enzyme denatured in the presence of 8 M urea. In 50 mM Tris-HCl buffer, pH 8.0, plus 1 mM EDTA, 1.05 ± 0.20 SH groups are found to be accessible, based on a subunit molecular mass of 37 kDa (Figure 3). Titration in 8 M urea reveals 2.5 SH groups/monomer. Since *Thermotoga maritima* GAPDH does not unfold completely in 8 M urea, this value suggests at least three sulfhydryl groups per monomer. The

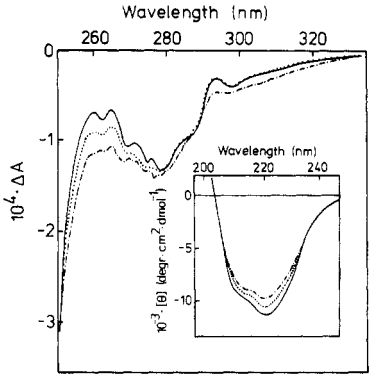

FIGURE 4: Aromatic CD in the near-UV of GAPDH from *Thermotoga maritima*. Instrumental base lines were subtracted, and accumulation of 32 spectra was performed numerically by computer. Enzyme concentration, 1.5 mg/mL; path length, 1 cm. (—) 20 °C; (···) 50 °C; (-·-) 80 °C. (Insert) Far-UV CD spectrum: (—) 20 °C; (···) 50 °C; (-·-) 80 °C.

modification of the first, most reactive SH group with DTNB leads to complete inactivation of the apoenzyme and to a ≈50% decrease in intrinsic fluorescence (see insert, Figure 3). This "fast" SH group may be assumed to be the essential cysteine residue in the active site of the enzyme. The quench in tryptophan fluorescence would then suggest that one of the two tryptophan residues is located in the vicinity of the active center.

In the calculation of the molar ratio of the various amino acids (Table II), it was assumed that 1 subunit consists of 332 amino acids, in accordance with the preliminary sequence analysis (Schultes et al., 1990).

Spectroscopic Characteristics. The ultraviolet absorption spectrum of GAPDH from *Thermotoga maritima* has its maximum at 276 nm and two shoulders at 268 and 282 nm. The A_{280}/A_{260} ratio of the purified enzyme (equilibrated with NAD^+) is 1.22.

The circular dichroism measurements were carried out with samples dialyzed against 50 mM KH_2PO_4, pH 8.0, plus 1 mM EDTA and 1 mM DTE. The CD spectrum is presented in Figure 4. The far-UV region is typical for an α-helical protein, characterized by a trough at 222 nm. At 50 °C ($[\theta]$ = 10 200 deg cm^2 dmol^{-1}), the calculated α-helix content is 32% (Chen et al., 1972); this corresponds to the value observed for other GAPDHs. The near-UV region of the CD spectrum reflects the asymmetric environment of the immobilized aromatic residues buried in the interior of the folded enzyme molecule. The overall features of this region of the CD spectrum of *Thermotoga* GAPDH are entirely different from those of the enzymes from rabbit (Boers et al., 1971) and *Bacillus stearothermophilus* (Suzuki & Imahori, 1973), both of which are closely similar with regard to their spectral properties.

Fluorescence emission of GAPDH from *Thermotoga maritima* at 20 °C shows a maximum at 326 nm (λ_{exc} = 280 nm) (Figure 3, insert). When the spectrum was monitored as a function of temperature, no increase in light scattering was detectable even after long-term incubation at 80 °C. This indicates that there is no thermal denaturation. Both the wavelength of the emission maximum (λ_{max}) and the relative fluorescence intensities change with temperature. As indicated by the blue-shift of λ_{max} ($\lambda_{max}^{45°C}$ = 326 nm → $\lambda_{max}^{65°C}$ = 321 nm), hydrophobic interactions around aromatic residues must be strengthened with increasing temperature; i.e., tryptophan residues appear to get even more buried into the apolar interior of the enzyme (Brand & Witholt, 1967). The observed decrease in fluorescence intensity with increasing temperature

Table III: Temperature Dependence of the Kinetic Parameters of D-Glyceraldehyde-3-phosphate Dehydrogenase from *Thermotoga maritima*: Oxidation of Glyceraldehyde 3-Phosphate at pH 8.0

	$K_{m,app}$ (μM)		
T (°C)	GAP	NAD^+	arsenate
40	440	18.6	460
50	360	37.0	710
60	400	78.8	

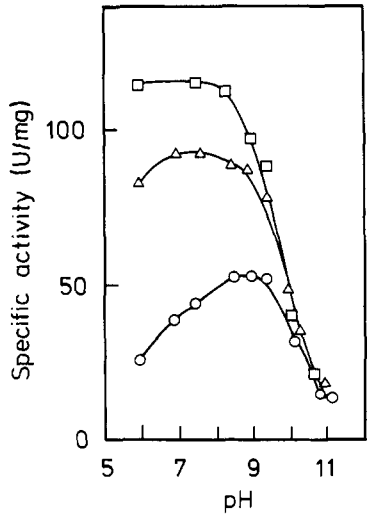

FIGURE 5: pH dependence of enzymatic activity of GAPDH from *Thermotoga maritima* at various temperatures. Oxidation of glyceraldehyde 3-phosphate was measured in Teorell buffer. (○) 40 °C, (△) 50 °C, (□) 60 °C.

is of the order of 40% for the temperature range from 10 to 80 °C. This value is below the decrease observed for the temperature dependence of the fluorescence of a mixture of tyrosine and tryptophan corresponding to the stoichiometry present in the *Thermotoga* enzyme, which is of the order of 80%.

Catalytic Properties. Due to the thermal instability of some of the substrates and the coenzyme, most kinetic experiments were carried out below 60 °C. Data are summarized in Table III. The oxidative reaction was followed at pH 8.0, corresponding to the pH optimum of the reaction at 40 °C which lies between pH 8.0 and 9.5. With increasing temperature, there is an increase in activity at pH 6–8. Beyond pH 9.5, deactivation is observed. The pH dependence of the oxidative reaction at varying temperatures is illustrated in Figure 5.

Temperature-Dependent Changes of Enzyme Activity and Conformation. The temperature dependence of the oxidative reaction for GAPDH from *Thermotoga maritima* and yeast was studied in the temperature range from 3 to 75 °C and from 3 to 50 °C, respectively (Figure 6); the corresponding Arrhenius diagram is given in the insert of the figure. As expected for complex enzyme reactions, no simple Arrhenius behavior is observed; linear approximation of the profiles at low and high temperatures leads to "breaks" at 37 and 18 °C, respectively. The limiting values of the corresponding "activation energies" for the enzyme from *Thermotoga maritima* are 78.6 kJ/mol for the lower temperature range and 32.8 kJ/mol for the upper one. A similar behavior has been reported for lactate dehydrogenase from *Thermus thermophilus* (Lakatos et al., 1978), as well as for GAPDH from *Thermoproteus tenax* (Hensel et al., 1987) and from *Methanothermus fervidus* (Fabry & Hensel, 1987). In all cases, the discontinuity is accompanied by similar changes in other thermodynamic quantities. To trace the conformational background of this "thermometer effect", the far- and near-UV

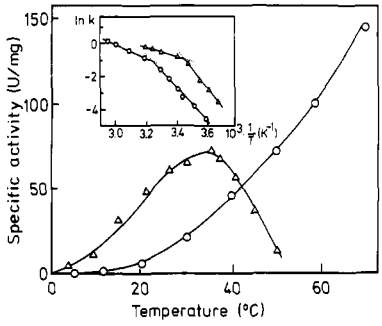

FIGURE 6: Temperature dependence of the specific activity of *Thermotoga maritima* (○) and yeast (△) D-glyceraldehyde-3-phosphate dehydrogenase. Oxidation of glyceraldehyde 3-phosphate. Insert: Arrhenius plot for the oxidation of glyceraldehyde 3-phosphate. Comparison of *Thermotoga maritima* (○) and yeast enzyme (△). Measurements were performed in 50 mM Teorell buffer, pH 8.5, plus 2 mM NAD^+, 20 mM arsenate, and 1 mM GAP.

circular dichroism and the intrinsic fluorescence emission were measured as a function of temperature. The experimental curves show a gradual decrease in the magnitude of the negative ellipticity in the far-UV with increasing temperature (Figure 4). This change can be explained by the temperature-dependent hypochromicity of the peptide chromophore. Despite the observed hypochromicity in the near-UV region, there is an increase in magnitude of the negative ellipticity of the aromatic chromophores, indicating a slight change in their environment. Proteins commonly show a red-shift of their fluorescence emission when temperature is increased. The effect can be attributed to an increase in mobility and accessibility of buried aromatic residues. In the case of *Thermotoga maritima*, the opposite effect is observed: increasing temperature leads to a small blue-shift accompanied by a small change in fluorescence intensity, indicating restricted flexibility of the aromatic side chains as a consequence of strengthened hydrophobic interactions.

Micro- and Macrostability. In order to quantify the thermal stability of GAPDH from *Thermotoga maritima*, the time course of the irreversible heat inactivation was monitored in the temperature range of 60–105 °C, and at various pH values. Since the heat inactivation kinetics are complex, the time required for 50% inactivation is used to characterize the process. The corresponding half-lives, $t_{1/2}$, for the holoenzyme at pH 5.0, 6.0, and 7.5, and 95 °C, are 25, >120, and 50 min, respectively. The enzyme exhibits maximal thermal stability at pH 6.0; at 100 °C, the half-life of the holoenzyme still exceeds 120 min. At pH 7.5, incubation at 70–85 °C results in an apparent activation of the enzyme. Beyond this temperature range, deactivation becomes significant. Plateau values at 90 and 95 °C point to heterogeneity of the enzyme; however, evidence from SDS–polyacrylamide gel electrophoresis and sedimentation analysis seems to indicate that deviations from homogeneity are insignificant.

The time course of the hydrogen–deuterium exchange of GAPDH from both *Thermotoga maritima* and yeast was followed at 25 °C in the range from pH 5.8 to 9.0. The ratio of unexchanged peptide hydrogen atoms was calculated as a function of time, pH, and temperature. The results were interpreted in terms of the EX_{II} mechanism (Hvidt & Nielsen, 1966), i.e., under the assumption that the fluctuations exposing buried H atoms are fast compared to the exchange rate constant (k_0) of the solvent-exposed peptide groups. k_0 is a function of pH and temperature (θ in degrees centigrade) and can be evaluated from the equation:

$$k_0 = (10^{-pH} + 10^{pH-6})10^{0.05(\theta-25)}\ s^{-1} \quad (2)$$

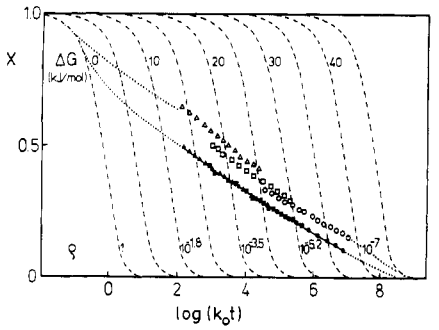

FIGURE 7: Hydrogen–deuterium exchange data, summarized in the form of relaxation spectra for GAPDH from *Thermotoga maritima* [measurements at pH* 5.8 (△), 7.5 (□), and 9.0 (○)] and GAPDH from yeast [pH* 5.9 (▲), 7.65 (■), and 8.9 (●)]. X is the fraction of unexchanged peptide hydrogens; k_0 was calculated according to eq 3. The relaxation spectra were extrapolated to zero and total exchanges (dotted lines). The dashed curves represent the exchange rate curves for hypothetical polypeptides that expose their peptide hydrogens in a cooperative way with the probability of ρ^i. $\overline{\Delta G}$ was calculated according to eq 4; the area enclosed between the two curves corresponds to the difference in $\overline{\Delta G}$ between the mesophilic and the thermophilic enzymes.

The exchange is supposed to proceed as a series of simultaneous first-order reactions:

$$X = n^{-1}\sum \exp(-\beta^i t) \qquad (3)$$

where n is the number of peptide hydrogens in the protein, $\beta^i = \rho^i k_0$ is the exchange rate constant of the ith peptide group, and ρ^i is the probability of finding the ith peptide hydrogen exposed to the solvent.

The kinetic data of the exchange are presented in Figure 7 as plots of X vs k_0t, rather than vs t. This representation is particularly useful for the comparison of measurements performed at varying pH, i.e., different values of k_0 (Willumsen, 1971).

Yeast GAPDH shows a continuous relaxation spectrum over the whole pH range from pH 5.8 to 9.0 which indicates that no detectable conformational changes occur as a function of pH. In the case of the enzyme from *Thermotoga maritima*, a slight increase in conformational flexibility is observed when the pH is raised from pH 5.8 to 7.5, and further to pH 9.0. The corresponding pH-dependent destabilization of the enzyme is also detectable in the above-mentioned results of the pH-dependent heat inactivation experiments. Compared to the yeast enzyme, the relaxation spectrum of GAPDH from *Thermotoga maritima* is shifted toward smaller ρ values. The shift corresponds to a 5.2 kJ/mol change in the activation free energy difference associated with the transconformation reactions involved in the exposure of buried hydrogens to the solvent.

Thermal analysis shows a pretransition at ≥95 °C. With the use of differential scanning microcalorimetry, the melting temperature, T_m, for the NAD$^+$-saturated enzyme (A_{280}/A_{260} = 1.1) was found to be 109 °C. Partial removal of the coenzyme by dialysis (A_{280}/A_{260} = 1.52) shifts the T_m to 107 °C. The thermal transition shows high cooperativity (Figure 8).

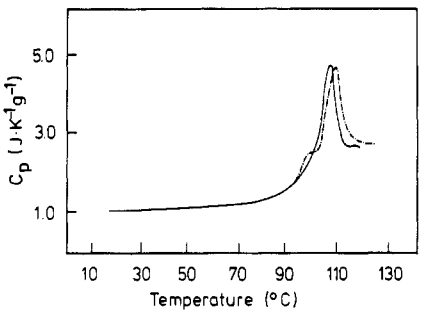

FIGURE 8: DSC thermogram of apo- and holo-GAPDH in 50 mM phosphate buffer, pH 6.0, plus 1 mM DTE/1 mM EDTA. Enzyme concentration, 1.0 mg/mL; heating rate, 1 °C/min. (—) Apoenzyme; (-·-) holoenzyme in the presence of 1 mM NAD$^+$.

Discussion

The genus *Thermotoga* represents the deepest known branch and the most slowly evolving line within the kingdom of eubacteria (Huber et al., 1986). GAPDH from this microorganism is an attractive choice to study the evolutionary aspect of enzyme structure, function, and stability. The enzyme has been isolated from a large number of organisms, both mesophiles and thermophiles. In the case of thermophilic microorganisms, it was found to exhibit intrinsic thermal stability. The most extreme thermophilic variants obtained so far were from the archaebacteria *Methanothermus fervidus* (Fabry & Hensel, 1987) and *Thermoproteus tenax* (Hensel et al., 1987). With its thermal transition at 109 °C, the enzyme from *Thermotoga maritima* exceeds the stability of any oligomeric protein investigated so far, thus confirming the observation that thermal adaptation of cells refers to their protein inventory as well. The expression level of GAPDH in *Thermotoga maritima* is exceedingly high (≈1.5 mg/g of wet cells). Its purification made use of conditions successfully applied for other GAPDHs in previous studies (Elödi & Szörényi, 1956; Harris & Perham, 1965; Fujita et al., 1976). This points to a high degree of similarity in the overall surface structure of the *Thermotoga* enzyme compared to other thermophilic and mesophilic GAPDHs. Actually, the detailed physicochemical characterization of the enzyme proves that it is closely similar to most eukaryotic and prokaryotic GAPDHs described so far. Its molecular mass is practically identical with the one reported for the homologous enzymes from *Bacillus stearothermophilus*, yeast, and higher organisms. As taken from their CD spectral properties, the conformation of the various homologous enzymes cannot differ significantly; similarly, sequence homologies point to a close relationship with respect to the three-dimensional topology of the molecule. The only feature which contrasts drastically to the previously described mesophilic and moderately thermophilic homologues is the extreme thermal stability of the *Thermotoga* enzyme. This cannot be correlated with the amino acid composition in a direct way.

Comparing available sequence data for the various homologous GAPDHs (Harris & Perham, 1965; Harris & Waters, 1976; Schultes et al., 1990), it becomes clear that the polypeptide chains can be aligned in an unambiguous way. The presence of an essential cysteine (Figure 3) and the conserved histidine (Schultes et al., 1990) clearly suggest that also from the mechanistic point of view the various homologues are closely related, if not identical.

Enhanced thermal stability of a protein molecule requires only marginal increments of free energy which do not exceed the equivalent of a few hydrogen bonds, hydrophobic interactions, or ion pairs, respectively. Previous attempts to explore general rules of thermal adaptation by a statistical comparison of thermophilic vs mesophilic proteins were unsuccessful due to the wide variability of sequences determining identical topologies (Jaenicke, 1981, 1987). In restricting their statistical analysis to a limited number of enzymes, or just one enzyme from various genera of bacteria with a wide range of optimum temperatures, Argos et al. (1979) and Menéndez-Arias and Argos (1989) deduced principles governing the "gross traffic"

of amino acid exchanges that determine thermostability. On one hand, cumulative effects of small improvements of intramolecular interactions at many locations in the three-dimensional structure were found to be essential; on the other hand, a hierarchy of preferred amino acid exchanges with the general tendency of increased hydrophobicity as a prerequisite of increased thermal stability became obvious.

Comparing the amino acid compositions of the various GAPDHs, it is obvious that previously suggested "traffic rules" of thermophilic adaptation can only partially be applied to the hyperthermophilic enzyme. The most significant exchange refers to Lys → Arg. Since most lysine residues are at the surface of the enzyme (Buehner et al., 1973; Walker et al., 1980), no significant steric hindrance is to be expected if lysine residues are changed into arginines. The stabilizing effect accompanying this exchange has been frequently reported (Tuengler & Pfleiderer, 1977; Lakatos et al., 1978; Väli et al., 1980). Apart from the Lys → Arg exchange, preferred exchanges refer to Ser → Thr, as well as an increase in Leu and Ile; Gly, Ala, and Val show average values also found in mesophiles. Without additional information with respect to the detailed three-dimensional structure of the enzyme, no ranking of the significance for the observed exchanges can be given.

From visual inspection of the presently available sequence data and preliminary computer simulations, the overall topology of the thermophilic enzyme closely resembles the known structures of the enzymes from lobster (Moras et al., 1975) and *Bacillus stearothermophilus* (Walker et al., 1980; Skarzynski et al., 1987). This holds also for the quaternary structure.

As found for all homologous GAPDHs investigated so far, the enzyme is a stable homotetramer. Sedimentation equilibrium experiments show that under meniscus depletion conditions even at 0 °C the oligomeric state prevails. Only at high rotor speeds and exceedingly low protein concentrations (<0.5 μM) the monomer becomes the predominant species.

Since there seem to be no major differences in the overall topology of the various homologous GAPDHs, we may conclude that replacements in the surface groups play a major role in the increased thermal stability of the thermophilic protein. Accordingly, alterations in the solvation of the polypeptide chain must be essential. In addition, the anomalous temperature dependences of the near-UV circular dichroism and the fluorescence spectra indicate that in *Thermotoga* GAPDH the packing of the aromatic side chains in their hydrophobic environment must be closer than in their mesophilic counterparts. With increasing temperature, further tightening of the hydrophobic interactions seems to occur. They compensate for the increased thermal agitation, thus preventing thermal unfolding.

Temperature-dependent NMR studies clearly indicated increased conformational flexibility of the native structure of proteins at elevated temperature (Campbell, 1977; Williams, 1978; Wüthrich, 1986). There are two counteracting requirements for a functional enzyme structure: (i) stability to provide the proper conformation required to recognize the ligands; (ii) flexibility to allow structural adjustment during binding and release of substrates, products, or allosteric modulators. The delicate balance between stability and flexibility results in the marginal free energy of stabilization of proteins under physiological conditions (Baldwin & Eisenberg, 1987).

The stability of a native enzyme can be characterized by the energy required for (cooperative) macrounfolding of the structure, while flexibility can be expressed in terms of the average Gibbs energy associated with local noncooperative microunfolding processes (Privalov & Tsalkova, 1979). These two properties were studied by following denaturation using scanning microcalorimetry, and measuring the kinetics of peptide hydrogen exchange. The question was how the obviously increased macrostability of the hyperthermophilic GAPDH is reflected in its conformational flexibility.

Hydrogen–deuterium exchange offers a good quantitative parameter to measure the average conformational flexibility of a macromolecule. $\overline{\Delta G}$, the average Gibbs free energy associated with the exposure of a peptide hydrogen to the solvent through noncooperative reversible fluctuations, can be calculated from the exchange data presented in Figure 7 by using the equation:

$$\overline{\Delta G} = -n^{-1}RT\sum \ln \rho^i \qquad (4)$$

The comparative measurements show that at 25 °C and pH 5.8–9.0 the microstability of GAPDH from *Thermotoga maritima* exceeds that of the yeast enzyme. The estimated difference is substantial: 5.2 kJ/mol; it can be explained by increased saturation of the structure with nonpolar contacts, as has been pointed out by Privalov and Khechinashvili (1974). The observed fluorescence and CD spectra are in accord with this explanation.

The decreased flexibility at 25 °C agrees with the increased activation energy of the catalyzed reaction below 37 °C for the thermophilic enzyme. This "thermometer effect" has been observed earlier with other thermophilic enzymes (Lakatos et al., 1978; Väli et al., 1980; Hensel et al., 1987; Fabry & Hensel, 1987) and has been related to the conformational flexibility of the protein (Vihinen, 1987; Jaenicke, 1988). Obviously, this is optimized at the respective physiological temperatures. Accordingly, the common nonlinear Arrhenius behavior (characteristic of multisubstrate enzymes) is shifted to higher temperatures when the thermophilic and mesophilic enzymes are compared (Figure 6).

Both fluorescence emission and near-UV circular dichroism reflect the changes in the functional state of the enzyme (Figures 3 and 4). Obviously, they are accompanied by conformational changes in the neighborhood of aromatic residues serving as local probes within the enzyme molecule. No quantitative evaluation of the spectral changes can be given because nothing is known with respect to the specific localization of the tryptophan and tyrosine residues within the tertiary and quaternary structure of the enzyme. As indicated by the absence of any deflections from the smooth base line in the calorimetric traces below ≈95 °C, the spectral effects can only be attributed to local changes in the structure.

The most obvious characteristic of extreme thermophilic oligomeric enzymes is the increased packing density of the amino acid side chains in the native quaternary structure and, subsequently, the decreased side-chain flexibility. Comparing homologous enzymes from thermophiles and mesophiles, their physicochemical and enzymatic properties converge at their respective optimum temperatures, supporting the view that under physiological conditions homologous enzymes occupy "corresponding states".

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Registry No. GAPDH, 9001-50-7; NAD, 53-84-9; D-glyceraldehyde 3-phosphate, 591-57-1; arsenate, 15584-04-0.

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